METHOD OF ENHANCING THE BIOLOGICAL ACTIVITY OF LIGANDS

This application is a U.S. National Stage Application of PCT International Application No. PCT/US99/30900, filed December 23, 199, which claims priority of U.S. Application No. 60/113,387, filed December 23, 1998. Throughout this application, various publications are cited. The disclosures of each and all of those publications are hereby incorporated by reference in their entireties into this application.

INTRODUCTION

The present invention provides for novel methods for producing novel fusion polypeptide ligands that have enhanced biological activity as compared to the polypeptide ligands in their native form. The invention also provides for nucleic acids useful for producing biologically active fusion polypeptide ligands, and the fusion polypeptide ligands themselves.

BACKGROUND OF THE INVENTION

The ability of polypeptide ligands to bind cells and thereby elicit a phenotypic response such as cell growth, survival or differentiation is often mediated through transmembrane tyrosine kinases. The extracellular portion of each receptor tyrosine kinase (RTK) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic. Binding of a ligand to the extracellular domain results in signal transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates (Mohammadi, et al.,1990, Mol. Cell. Biol., 11: 5068-5078; Fantl, et al., 1992, Cell, 69:413-413).

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13:443-447; Ullrich and Schlessinger, 1990, Cell, 61:203-212; Schlessinger and Ullrich, 1992, Neuron 9:383-391); molecular interactions between dimerizing cytoplasmic domains lead to activation of kinase function. In some instances, such as the growth factor platelet derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al. , 1988, Science, 240: 1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of EGF, the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem., 259:14631-14636).

Throughout the history of the biotechnology industry, many novel genes and associated proteins have been identified by virtue of their sequence homology with known genes. Many such proteins are purported to be receptors, but since their cognate ligands have not been identified, they are referred to as orphan receptors. The screening of many of these orphan receptors often leads to the identification of ligands that are capable of binding to the receptor, although the binding is often not associated with activation of any intracellular kinases or any other phenotypic change. Such was the case for members of the Eph receptor family. For sake of clarity, applicants incorporate by reference herein a letter cited as Eph Nomenclature Committee, 1997, published in Cell vol. 90: 403-403 (1997) which sets forth a nomenclature for the Eph Receptor and Eph Ligand Families.

Little, if any, biological activity had been observed in response to binding of a ligand to an Eph family receptor prior to the discovery as set forth in U.S. Patent No. 5,747,033 issued May 5, 1998. That patent describes the concept of "clustering" whereby the soluble domains of ligands were combined to create multimers capable of activating the cognate receptors. Applicants have now extended the concept of clustering to additional ligands outside the Eph family, for example, the Tie-2 receptor ligands known as the angiopoietins, and have also discovered that this method for production of homogeneous forms of clustered ligands is broadly applicable to improve

the affinity and/or increase the activity of a ligand as compared to the native form of the ligand.

Angiopoietin-1 (Ang) is one of two known ligands for the Tie-2 receptor and has been shown to be an agonist for Tie-2 (Davis, et al, 1996, Cell 87:1161-1169), whereas the second known ligand, angiopoietin-2, has been shown to be a naturally occurring antagonist of the Tie-2 receptor (Maisonpierre, et al., 1997, Science 277:55-60). Ang1* is a mutant form of angiopoietin-1 that comprises the N-terminal domain of angiopoietin-2 fused to the coiled-coil domain and the fibrinogen domain of angiopoietin-1 and that has a Cys to Ser mutation at amino acid 245. Ang1* has been shown to be a potent agonist for the Tie-2 receptor.

Experiments with mutants of angiopoietin-1 and angiopoietin-2 have demonstrated that the fibrinogen domains (FD) are the receptor-binding domains, and that dimerized versions of, for example Ang-1-FD-Fc, (i.e., the fibrinogen domain of Ang-1 fused to an Fc domain), can bind to the Tie-2 receptor with much higher affinity than monomeric Ang-1-FD (dimerization occurs due to the interaction between the Fc components of adjacent molecules). However, Ang-1-FD-Fc is not able to induce phosphorylation (activate) the Tie-2 receptor on endothelial cells unless it is further clustered with goat anti-human Fc antibodies (Jackson Immunoresearch). For this reason, mutant versions of Ang-1-FD and Ang-2-FD (i.e., the fibrinogen domain of Ang-2) were designed that were intrinsically more highly clustered.

SUMMARY OF THE INVENTION

The present invention provides for novel, biologically active, soluble forms of polypeptide ligands that bind to receptors on cells. Such polypeptide ligands are useful in promoting a differential function and/or influencing the phenotype, such as growth and/or proliferation, of receptor-bearing

cells. The invention also provides for nucleic acids encoding such polypeptide ligands, and both prokaryotic and eukaryotic expression systems for producing such polypeptide ligands. According to the invention, soluble forms of the polypeptide ligands described herein may be used to promote biological responses in receptor-expressing cells. In particular, a general method is described herein which produces fusion polypeptide ligands that may then be clustered, which functions to make otherwise inactive soluble polypeptide ligands biologically active, or which enhances the biological activity of polypeptide ligands that, absent such clustering, would have lower levels of biological activity. This method may be used to cluster a plurality of (more than one) receptor binding domains from any ligand which has improved affinity and/or increased activity (i.e. signaling ability) when clustered as compared to the native form of the ligand.

DESCRIPTION OF THE FIGURES

Figure 1A-1E - Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-FD-Fc.

Figure 2A-2E - Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-FD-Fc.

Figure 3A-3E - Nucleic acid sequence and deduced amino acid sequence of Ang-1-FD-Fc-FD.

Figure 4A-4E - Nucleic acid sequence and deduced amino acid sequence of Ang-2-FD-Fc-FD.

Figure 5 - Molecular Weight Analysis of Ang-1-FD-Fc-FD protein. SDS PAGE analyses showing a band running at about 210kD under non-reducing conditions (lane 3) and a band running at about 85kD under reducing conditions (lane 7).

Figure 6 - Light scatter analysis to confirm the molecular weight of Ang-1-FD-Fc-FD and to determine whether or not the protein is a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent is monitored with an on line light scattering detector and a refractive index and/or a UV detector. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) is used to calculate the protein concentration. The molecular weight of protein is then calculated from the angular dependence of light scattering. The molecular weight of the dimeric protein appears to be approximately 200kD and presence of a single peak implies that the protein solution is homogenous.

Figure 7 - Molecular Weight Analysis of Ang-2-FD-Fc-FD. SDS PAGE analyses showing a band running at about 200kD under non-reducing conditions (lanes 7 and 8) and a band running at about 88kD under reducing conditions (lanes 3 and 4).

Figure 8 - Light scatter analysis to confirm the molecular weight of Ang-2-FD-Fc-FD and to determine whether or not the protein is a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent is monitored with an on line light scattering detector and a refractive index and/or a UV detector. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) is used to calculate the protein concentration. The molecular weight of protein is then calculated from the angular dependence of light scattering. The molecular weight of the dimeric protein appears to be approximately 171kD and presence of a single peak implies that the

protein solution is homogenous.

Figure 9 - Ang1*-mediated vs. Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. A standard phosphorylation assay revealed that Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926.

Figure 10 - Ability of Ang-2-FD-Fc-FD to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells. In a standard phosphorylation assay, Ang-2-FD-Fc-FD is able to block Ang1* stimulation of the Tie-2 receptor when it is present in at least a 10-15 fold molar excess of Ang1*.

Figure 11 - Ability of angiopoietin-2 to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells. In a standard phosphorylation assay, at a 20 fold molar excess, angiopoietin-2 is not able to reduce the Ang1*-mediated phosphorylation level to 50%. This result, coupled with the results described in Figure 10 implies that Ang-2-FD-Fc-FD is a more potent inhibitor of Ang1*-mediated Tie-2 receptor phosphorylation than angiopoietin-2.

Figure 12 - Ability of Ang-2-FD-Fc-FD to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells. In a standard phosphorylation assay, it is shown that while there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, Ang-2-FD-Fc-FD seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 10.

Figure 13 - Ability of angiopoietin-2 to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells. In a standard phosphorylation assay, it is shown that there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, but, like Ang-2-FD-Fc-FD, angiopoietin-2 seems to be more effective at

blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 11.

Figure 14A-14E - Nucleic acid sequence and deduced amino acid sequence of Ephrin-B1-Ephrin-B1-Fc.

Figure 15A-15E - Nucleic acid sequence and deduced amino acid sequence of Ephrin-B2-Ephrin-B2-Fc.

Figure 16 - Comparison of Ephrin-B1-Fc, Ephrin-B1-Ephrin-B1-Fc, Ephrin-B2-Fc and Ephrin-B2-Ephrin-B2-Fc in standard EphB2 phosphorylation assays. COS cells were serum-starved and then left untreated (UT), lane 1, or were treated with unclustered and clustered Ephrin-B1-Fc (Efn-B1), lanes 2 and 3. COS cells were also treated with unclustered and clustered Ephrin-B1-Ephrin-B1-Fc (Efn-B1 DD), lanes 4 and 5. In addition cells were likewise treated with unclustered and clustered Ephrin-B2-Fc (Efn-B2), lanes 6 and 7 and with unclustered and clustered Ephrin-B2-Ephrin-B2-Fc (Efn-B2 DD), lanes 8 and 9. The extent of EphB2 phosphorylation was assessed by anti-phosphotyrosine western blotting (upper panels) and the relative amounts of EphB2 in each lane was determined by anti-EphB2 western blotting (lower panels).

Figure 17 - Ang1*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were stimulated with 0.4 μg/ml Ang1* or 0.2 μg/ml or 0.4 μg/ml stable CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells.

Figure 18 - Ability of stable CHO clone-derived Ang-2-FD-Fc-FD to block stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells. EAhy926 cells were treated with 0.2

μg/ml of the Tie-2 agonist Ang-1-FD-Fc-FD and 2 μg/ml, 4 μg/ml, 8 μg/ml or 16 μg/ml of stable CHO clone-derived Ang-2-FD-Fc-FD. Ang-2-FD-Fc-FD is able to block or stable CHO clone-derived Ang-1-FD-Fc-FD stimulation of the Tie-2 receptor when it is present in at least a 40 fold molar excess of stable CHO clone-derived Ang-1-FD-Fc-FD.

DETAILED DESCRIPTION OF THE INVENTION

As described in greater detail below, applicants have discovered a method for "clustering" polypeptide ligands, which functions to make otherwise inactive soluble polypeptide ligands biologically active, or which enhances the biological activity of polypeptide ligands that, absent such clustering, would have lower levels of biological activity. This method may be used to cluster a plurality of (more than one) receptor binding domains from any ligand which has improved affinity and/or increased activity (i.e. signaling ability) when clustered as compared to the native form of the ligand.

The present invention provides for a nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises a first subunit comprising at least one copy of the receptor binding domain of a ligand, the first subunit being fused to the N-terminal end of a multimerizing component, said multimerizing component being fused at its C-terminal end to a second subunit comprising at least one copy of the receptor binding domain of a ligand.

In one embodiment of the invention, the receptor binding domains of the first and second subunits are copies of the receptor binding domain of the same ligand. The first and second subunits may each have one or more than one copy of the receptor binding domain of the ligand. In specific embodiments of the invention, the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2. Alternatively, the

receptor binding domain is from a ligand selected from the group consisting of the EPH family of ligands (i.e., the ephrins).

In another embodiment of the invention, the receptor binding domains of the first subunit are copies of the receptor binding domain of a different ligand from the receptor binding domains of the second subunit. For example, the first subunit may comprise the receptor binding domain of an angiopoietin and the second subunit may comprise the receptor binding domain of vascular endothelial growth factor (VEGF). Alternatively, the first subunit may comprise the receptor binding domain of VEGF and the second subunit may comprise the receptor binding domain an angiopoietin. Still further, the first and second subunits may each have one or more than one copy of the receptor binding domain of their respective ligand.

By "receptor binding domain" what is meant is the minimal portion of the ligand that is necessary to bind its receptor.

In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(ΔC1).

The present invention also provides for fusion polypeptides encoded by the nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are described in European Patent Application of ZymoGenetics, Inc., Publication No. EP 0 721 983 A1 published 17 July 1996

and include S. cerevisiae repressible acid phosphatase (Mizunaga et al., 1988, J. Biochem. (Tokyo) 103:321-326); the S. cerevisiae type 1 killer preprotoxin (Sturley et al., 1986, EMBO J. 5:3381-3390); the S. calsbergensis alpha galactosidase melibiase (Sumner-Smith, et al., 1985, Gene 36:333-340); and the Neurospora crassa ornithine decarboxylase (Digangi, et al., 1987, J. Biol. Chem. 262:7889-7893). Sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); the S. cerevisiae SUC2 gene (Carlson et al., 1983, Mol. Cell. Biol. 3:439-447); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS or CHO cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The fusion polypeptides useful for practicing the present invention may be prepared by expression in a prokaryotic or eukaryotic expression system.

The recombinant gene may be expressed and the polypeptide purified utilizing any number of methods. The gene may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The fusion polypeptides may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The present invention also provides for a nucleic acid encoding a fusion polypeptide wherein the fusion polypeptide comprises more than one copy of the receptor binding domain of a ligand in tandem, and wherein either the N-terminal or the C-terminal receptor binding domain is also fused to a multimerizing component. In one embodiment of the invention, the receptor binding domains are fused contiguously. In another embodiment of the invention, the receptor binding domains are from a ligand that is not a member of the EPH family of ligands (i.e., not an ephrin). In specific embodiments of the invention, the receptor binding domain is the fibrinogen domain of angiopoietin-1 or angiopoietin-2. In an alternative embodiment, the receptor binding domain is from vascular endothelial growth factor (VEGF). In another embodiment, the receptor binding domain is from an ephrin.

By "receptor binding domain" what is meant is the minimal portion of the ligand that is necessary to bind its receptor.

In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(ΔC1).

The present invention also provides for fusion polypeptides encoded by the nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are described in European Patent Application of ZymoGenetics, Inc., Publication No. EP 0 721 983 A1 published 17 July 1996 and include S. cerevisiae repressible acid phosphatase (Mizunaga et al., 1988, J. Biochem. (Tokyo) 103:321-326); the S. cerevisiae type 1 killer preprotoxin (Sturley et al., 1986, EMBO J. 5:3381-3390); the S. calsbergensis alpha galactosidase melibiase (Sumner-Smith, et al., 1985, Gene 36:333-340); and the Neurospora crassa ornithine decarboxylase (Digangi, et al., 1987, J. Biol. Chem. 262:7889-7893). Sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); the S. cerevisiae SUC2 gene (Carlson et al., 1983, Mol. Cell. Biol. 3:439-447); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is

operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS or CHO cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The fusion polypeptides useful for practicing the present invention may be prepared by expression in a prokaryotic or eukaryotic expression system. The recombinant gene may be expressed and the polypeptide purified utilizing any number of methods. The gene may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The fusion polypeptides may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The Examples describe the preparation of novel polypeptide ligands that comprise a receptor binding domain of a member of the Eph (Eph transmembrane tyrosine kinase family ligands) family of ligands or of a

member of the angiopoietin family of ligands that can bind the Tie-2 receptor.

For a description of novel Eph family ligands, methods of making and using them, as well as the sequences of EHK-1L, B61 and ELK-L, together with a description of a method of enhancing the biological activity of EPH family ligands by clustering them, applicants refer to U.S. Patent No. 5,747,033 issued on May 5, 1998 which is hereby incorporated by reference in its entirety. Applicants further refer to International Application PCT/US93/10879, published as WO 94/11020 on May 26, 1994; and International Application PCT/US96/17201 published as WO 97/15667 entitled "Biologically Active EPH Family Ligands" each of which is hereby incorporated by reference in its entirety.

As has been previously reported, a family of ligands for the TIE-2 receptor has been discovered and named the Angiopoietins. This family, consisting of TIE-2 ligand 1 (Ang-1); TIE-2 ligand 2 (Ang-2); TIE ligand 3 (Ang-3); and TIE ligand 4 (Ang-4) has been extensively characterized. For a description of the cloning, sequencing and characterization of the angiopoietins, as well as for methods of making and uses thereof, including the production and characterization of modified and chimeric ligands thereof, reference is hereby made to the following publications, each of which is incorporated by reference herein in its entirety: U.S. Patent No. 5,521,073 issued May 28, 1996; U.S. Patent No. 5,643,755 issued July 1, 1997; U.S. Patent No. 5,650,490 issued July 22, 1997; U.S. Patent No. 5,814,464 issued September 29, 1998; U.S. Patent No. 5,879,672 issued March 9, 1999; U.S. Patent No. 5,851,797 issued December 22, 1998; PCT International Application entitled "TIE-2 Ligands Methods of Making and Uses Thereof," published as WO 96/11269 on 18 April 1996 in the name of Regeneron Pharmaceuticals, Inc.; PCT International Application entitled "TIE-2 Ligands Methods of Making and Uses Thereof,"published as WO 96/31598 on 10 October 1996 in the name of Regeneron Pharmaceuticals, Inc.; PCT International Application entitled

"TIE-2 Receptor Ligands (TIE Ligand-3; TIE Ligand-4) And Their Uses," published as WO 97/48804 on 24 December 1997 in the name of Regeneron Pharmaceuticals, Inc; and PCT International Application entitled "Modified TIE-2 Receptor Ligands," published as WO 98/05779 on 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc.

When used herein, fusion polypeptide includes functionally equivalent molecules in which amino acid residues are substituted for residues within the sequence resulting in a silent or conservative change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent or conservative alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are proteins or fragments or derivatives thereof which exhibit the same or similar biological activity and derivatives which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Cells that express the fusion polypeptides of the invention are genetically engineered to produce them by, for example, transfection, transduction, electroporation, or microinjection.

The present invention encompasses the nucleic acid sequences encoding the fusion polypeptides of the invention, as well as sequences that hybridize under stringent conditions to nucleic acid sequences that are complementary to the nucleic acid sequences of the invention. Stringent conditions are set forth in, for example, Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2 ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989). In addition, the present invention encompasses nucleic acid sequences that are different from the nucleic acid sequences of the invention but which nevertheless encode the fusion polypeptides of the invention due to the degeneracy of the genetic code.

In addition, the present invention contemplates use of the fusion polypeptides described herein in tagged forms.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the fusion polypeptides of the invention using appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence encoding the fusion polypeptides of the invention may be regulated by a second nucleic acid sequence so that the fusion polypeptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the fusion polypeptides described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the fusion polypeptide include, but are not limited to the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980,

Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the b-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene

control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising Eph fusion polypeptide encoding or angiopoietin fusion polypeptide encoding nucleic acids as described herein, are used to transfect the host and thereby direct expression of such nucleic acid to produce fusion polypeptides which may then be recovered in biologically active form. As used herein, a biologically active form includes a form capable of binding to the relevant receptor and causing a differentiated function and/or influencing the phenotype of the cell expressing the receptor. Such biologically active forms would, for example, induce phosphorylation of the tyrosine kinase domain of the Ehk-1, Elk, or Tie2 receptor, or stimulation of synthesis of cellular DNA.

Expression vectors containing the nucleic acid inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign nucleic acids inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted nucleic acid sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign nucleic acid sequences in the vector. For example, if an efl nucleic acid sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign nucleic acid product expressed by the recombinant. Such assays can be based, for example, on the physical or

functional properties of the nucleic acid product of interest, for example, by binding of a ligand to a receptor or portion thereof which may be tagged with, for example, a detectable antibody or portion thereof or binding to antibodies produced against the protein of interest or a portion thereof.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the ephrin or angiopoietin fusion polypeptide as described herein.

The ephrin fusion polypeptides of the invention may be useful in methods of treating a patient suffering from a neurological disorder comprising treating the patient with an effective amount of the ephrin fusion polypeptide.

For example, the Elk receptor is expressed primarily in brain. Accordingly, it is believed that an Elk binding ephrin fusion polypeptide ligand will support the induction of a differential function and/or influence the phenotype, such as growth and/or survival of neural cells that express this receptor.

The present invention also provides for pharmaceutical compositions comprising the ephrin fusion polypeptide in a suitable pharmacologic carrier. The compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

As our understanding of neurodegenerative disease/neurotrauma becomes clearer, it may become apparent that it would be beneficial to decrease the effect of endogenous Efl-6. Therefore, in areas of nervous system trauma, it may be desirable to provide Efl-6 antagonists, including, but not limited to,

fusion polypeptide forms of Efl-6 which may compete with cell-bound ligand for interaction with Elk receptor. It may be desirable to provide such antagonists locally at the injury site rather than systemically. Use of an Efl-6 antagonist providing implant may be desirable.

Alternatively, certain conditions may benefit from an increase in Efl-6 responsiveness. It may therefore be beneficial to increase the number or binding affinity of Efl-6 in patients suffering from such conditions.

The invention herein further provides for the development of a fusion polypeptide, as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the TIE-2 receptor. Such molecules may be used in a method of treatment of the human or animal body, or in a method of diagnosis.

Because TIE-2 receptor has been identified in association with endothelial cells and, as was previously demonstrated, blocking of agonists of the receptor such as TIE-2 ligand 1 (Ang-1) has been shown to prevent vascularization, applicants expect that TIE-2 agonist fusion polypeptides of the invention may be useful for the induction of vascularization in diseases or disorders where such vascularization is indicated. Such diseases or disorders would include wound healing, ischemia and diabetes. The ligands may be tested in animal models and used therapeutically as described for other agents, such as vascular endothelial growth factor (VEGF), another endothelial cell-specific factor that is angiogenic. Ferrara, et al. U.S. Patent No. 5,332,671 issued July 26, 1994. The Ferrara reference, as well as other studies, describe in vitro and in vivo studies that may be used to demonstrate the effect of an angiogenic factor in enhancing blood flow to ischemic myocardium, enhancing wound healing, and in other therapeutic settings wherein neoangiogenesis is desired. [see Sudo, et al., European Patent Application 0 550 296 A2 published July 7, 1993; Banai, et al. Circulation 89:2183-2189 (1994); Unger, et al. Am. J. Physiol. 266:H1588-H1595

(1994); Lazarous, et al. Circulation 91:145-153 (1995)]. According to the invention, the agonist fusion polypeptides may be used alone or in combination with one or more additional pharmaceutically active compounds such as, for example, VEGF or basic fibroblast growth factor (bFGF).

Conversely, antagonists of the TIE-2 receptor, such as TIE-2 receptorbodies or TIE-2 ligand 2 (Ang-2) as described in Example 9 in International Publication No. WO 96/31598 published 10 October 1996, have been shown to prevent or attenuate vascularization, and are thus expected to be useful in preventing or attenuating, for example, tumor growth. Similarly then, TIE-2 antagonist fusion polypeptides of the invention would also be useful for those purposes. These antagonists may be used alone or in combination with other compositions, such as anti-VEGF antibodies, that have been shown to be useful in treating conditions in which the therapeutic intent is to block angiogenesis.

For example, applicants have determined that TIE-2 ligands are expressed in cells within, or closely associated with, tumors. For example, TIE-2 ligand 2 (Ang-2) appears to be tightly associated with tumor endothelial cells. Accordingly, TIE-2 antagonist fusion polypeptides of the invention may also be useful in preventing or attenuating, for example, tumor growth.

In other embodiments, the TIE-2 agonist fusion polypeptides of the invention described herein may be used as hematopoietic factors. A variety of hematopoietic factors and their receptors are involved in the proliferation and/or differentiation and/or migration of the various cells types contained within blood. Because the TIE-2 receptors are expressed in early hematopoietic cells, the TIE-2 ligands are expected to play a comparable role in the proliferation or differentiation or migration of these cells. Thus, for example, TIE-2 agonist fusion polypeptide compositions may be prepared, assayed, examined in in vitro and in vivo biological systems and

used therapeutically as described in any of the following: Sousa, U.S. Patent No. 4,810,643, Lee, et al., Proc. Natl. Acad. Sci. USA 82:4360-4364 (1985) Wong, et al. Science, 228:810-814 (1985); Yokota, et al. Proc. Natl. Acad. Sci (USA) 81:1070 (1984); Bosselman, et al. WO 9105795 published May 2, 1991 entitled "Stem Cell Factor" and Kirkness, et al. WO 95/19985 published July 27, 1995 entitled "Haemopoietic Maturation Factor". Accordingly, the fusion polypeptides may be used to diagnose or treat conditions in which normal hematopoiesis is suppressed, including, but not limited to anemia, thrombocytopenia, leukopenia and granulocytopenia. In a preferred embodiment, the fusion polypeptides may be used to stimulate differentiation of blood cell precursors in situations where a patient has a disease, such as acquired immune deficiency syndrome (AIDS) which has caused a reduction in normal blood cell levels, or in clinical settings in which enhancement of hematopoietic populations is desired, such as in conjunction with bone marrow transplant, or in the treatment of aplasia or myelosuppression caused by radiation, chemical treatment or chemotherapy.

The fusion polypeptides of the present invention may be used alone, or in combination with another pharmaceutically active agents such as, for example, cytokines, neurotrophins, interleukins, etc. In a preferred embodiment, the fusion polypeptides may be used in conjunction with any of a number of factors which are known to induce stem cell or other hematopoietic precursor proliferation, or factors acting on later cells in the hematopoietic pathway, including, but not limited to, hemopoietic maturation factor, thrombopoietin, stem cell factor, erythropoietin, G-CSF, GM-CSF, etc.

In an alternative embodiment, TIE-2 receptor antagonist fusion polypeptides are used to diagnose or treat patients in which the desired result is inhibition of a hematopoietic pathway, such as for the treatment of myeloproliferative or other proliferative disorders of blood forming organs

such as thrombocythemias, polycythemias and leukemias. In such embodiments, treatment may comprise use of a therapeutically effective amount of the fusion polypeptides as described herein.

Effective doses useful for treating these or other diseases or disorders may be determined using methods known to one skilled in the art [see, for example, Fingl, et al., The Pharmacological Basis of Therapeutics, Goodman and Gilman, eds. Macmillan Publishing Co., New York, pp. 1-46 ((1975)]. Pharmaceutical compositions for use according to the invention include the fusion polypeptides described above in a pharmacologically acceptable liquid, solid or semi-solid carrier, linked to a carrier or targeting molecule (e.g., antibody, hormone, growth factor, etc.) and/or incorporated into liposomes, microcapsules, and controlled release preparation prior to administration *in vivo*. For example, the pharmaceutical composition may comprise a fusion polypeptide in an aqueous solution, such as sterile water, saline, phosphate buffer or dextrose solution. Alternatively, the active agents may be comprised in a solid (e.g. wax) or semi-solid (e.g. gelatinous) formulation that may be implanted into a patient in need of such treatment. The administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an implanted device.

Administration may result in the distribution of the active agent of the invention throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of agent may be desirable. In some situations, an implant containing active agent may be placed in or near the lesioned area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

The present invention also provides for pharmaceutical compositions comprising the fusion polypeptides described herein, in a pharmacologically acceptable vehicle. The compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

EXAMPLES

Angiopoietin ligands:

As described *supra*, experiments with mutants of Ang-1 and Ang-2 have demonstrated that the fibrinogen domains (FD) are the receptor-binding domains, and that dimerized versions (dimerization occurs due to the interaction between the Fc components of adjacent molecules), for example Ang-1-FD-Fc, can bind to the Tie-2 receptor with much higher affinity than monomeric Ang-1-FD. However, Ang-1-FD-Fc is not able to induce phosphorylation (activate) the Tie-2 receptor on endothelial cells unless it is further clustered with goat anti-human Fc antibodies (Jackson Immunoresearch). For this reason, mutant versions of Ang-1-FD and Ang-2-FD were designed that were intrinsically more highly clustered.

Two general types of nucleic acid molecules were constructed. The first type consisted of two tandem copies of Ang-1-FD fused to an Fc tag, thus leading

to a secreted polypeptide molecule that is dimeric with respect to the Fc tag but tetrameric with respect to Ang-1-FD. Similarly, two tandem copies of Ang-2-FD fused to an Fc tag constituted the angiopoietin-2 version of this type of construct. These molecules were designated Ang-1-FD-FD-Fc and Ang-2-FD-FD-Fc, respectively.

In the second type of nucleic acid molecule constructed, two copies of Ang-1-FD were connected by an Fc tag bridging between them, thus creating the structure Ang-1-FD-Fc-FD that is still dimeric with respect to the Fc, as well as tetrameric with respect to Ang-1-FD. An angiopoietin-2 version was also constructed and these two molecules were designated Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD, respectively.

For either type of construct, similar properties were observed: unlike dimeric Ang-1-FD-Fc, which fails to activate Tie-2 in endothelial cells, both Ang-1-FD-FD-Fc and Ang-1-FD-Fc-FD could readily activate Tie-2 in endothelial cells, with a potency comparable to that of the native ligand. Also, like native angiopoietin-2, Ang-2-FD-Fc-FD could antagonize angiopoietin-1 activity with a potency that is comparable to that of native angiopoietin-2, and with much greater potency than the marginally antagonistic activity of the Ang-2-FD-Fc dimer.

Construction of mutant angiopoietin nucleic acid molecules.

All of the following nucleic acid molecules were constructed by standard recombinant DNA techniques (See e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), sequence-verified by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and subcloned into the mammalian expression vector pMT21 (Genetics

Institute, Inc.) with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5′ end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

Example 1: Construction of the Ang-1-FD-FD-Fc, Ang-2-FD-FD-Fc, Ang-1-FD-Fc-FD, and Ang-2-FD-Fc-FD nucleic acid molecules.

Ang-1-FD-FD-Fc: Ang-1-FD-FD-Fc consists of a trypsin signal sequence at its amino terminus to allow for secretion (bases 1-45 of Figure 1A) followed by the angiopoietin-1 fibrinogen domain (FD) (bases 46-690 of Figure 1A-Figure 1B), a short bridging sequence consisting of the amino acids Gly-Pro Ala-Pro (bases 691-702 of Figure 1B), a second angiopoietin-1 FD (bases 703-1750 of Figure 1B-Figure 1D), another bridging sequence consisting of the amino acids Gly-Pro-Gly (bases 1351-1359 of Figure 1D), and the coding sequence for the Fc portion of human IgG1 (bases 1360-2058 of Figure 1D-Figure 1E).

Ang-2-FD-FD-Fc: The Ang-2-FD-FD-Fc nucleic acid molecule was similarly constructed. It consists of a trypsin signal sequence (bases 1-45 of Figure 2A), an angiopoietin-2 FD (bases 46-690 of Figure 2A- Figure 2B), a bridging amino acid sequence Gly-Gly-Pro-Ala-Pro (bases 691-705 of Figure 2B), a second angiopoietin-2 FD (bases 706-1353 of Figure 2B-Figure 2D), another bridging amino acid sequence Gly-Pro-Gly (bases 1354-1362 of Figure 2D), and the coding sequence for the Fc portion of human IgG1 (bases 1363-2061 of Figure 2D-Figure 2E.

Ang-1-FD-Fc-FD: The Ang-1-FD-Fc-FD consists of a trypsin signal sequence (bases 1-45 of Figure 3A), an angiopoietin-1 FD (bases 46-690 of Figure 3A-3B), the bridging amino acid sequence Gly-Pro-Gly (bases 691-699 of Figure

3B), the coding sequence for the Fc portion of human IgG1 (bases 700-1395 of Figure 3B-3D), another bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1396-1419 of Figure 3D), and a second angiopoietin-1 FD (bases 1420-2067 of Figure 3D-Figure 3E).

Ang-2-FD-Fc-FD: The Ang-2-FD-Fc-FD nucleic acid molecule consists of a trypsin signal sequence (bases 1-45 of Figure 4A), an angiopoietin-2 FD domain (bases 46-690 of Figure 4A-Figure 4B), the bridging amino acid sequence Gly-Gly-Pro-Gly (bases 691-702 of Figure 4B), the coding sequence for the Fc portion of human IgG1 (bases 703-1398 of Figure 4B- Figure 4D), the bridging amino acid sequence Gly-Gly-Gly-Gly-Ser-Gly-Ala-Pro (bases 1399-1422 of Figure 4D), and a second angiopoietin-2 FD (bases 1423-2067 of Figure 4D-Figure 4E).

Example 2: Characterization of Ang-1 FD-Fc-FD protein.

Molecular Weight Analysis: The predicted molecular weight for Ang-1-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form (with respect to the Fc) has a predicted weight of 76,349. In addition, there are three predicted N-linked glycosylation sites, approximately 2500 MW/site, that could potentially increase the molecular weight of the monomeric protein to 83,849. Due to the interaction between the Fc components of adjacent molecules, the protein actually exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,698. Subsequent SDS PAGE analyses of COS cell-derived protein described *infra* confirmed these approximate molecular weights, with a band running at about 210kD under non-reducing conditions and a band running at about 85kD under reducing conditions (Figure 5). Light scatter analysis was performed to further confirm the molecular weight and, more importantly, determine whether or not the protein was a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To

determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent was monitored with an on line light scattering detector and a refractive index and/or a UV detector. The light scattering detector is a MiniDawn laser light scattering detector was from Wyatt Technology Corporation (Santa Barbara, CA). This instrument measures static light at three different angles. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) was used to calculate the protein concentration based on either dn/dc (dn = change of refractive index; dc = concentration) or the extinction coefficient of the protein. The molecular weight of protein is then calculated from the angular dependence of light scattering. Figure 6 shows the results of this analysis using COS cell-derived protein. The molecular weight of the dimeric protein appears to be approximately 200kD and presence of a single peak implies that the protein solution is, in fact, homogenous.

Expression Level in COS Cells: COS cell supernatant containing recombinant Ang-1-FD-Fc-FD protein was generated by transiently transfecting COS cells with the Ang1-FD-Fc-FD DNA construct described *supra*. All transfections were performed using standard techniques known in the art. The COS cell supernatant was analyzed using Biacore technology (Pharmacia, Inc.) to quantitate the amount of Ang-1-FD-Fc-FD protein present in the supernatant. This analysis resulted in an RU value of 765, which is equivalent to 0.9mg of recombinant protein/liter of COS cell supernatant. These values represent very high levels of expression.

Purification of COS Supernatants: Because the Ang-1-FD-Fc-FD protein contains an Fc domain, purification is relatively simple and straight forward using standard Protein A column chromatography (Pharmacia, Inc.) followed by standard size exclusion chromatography (Pharmacia, Inc.). In fact, the relative ease of purification of the Ang-1-FD-Fc-FD protein gives it a distinct advantage over the parent protein, angiopoietin-1, from which

it is derived, and the mutant version of angiopoietin-1 called Ang1* that consists of the N-terminal of angiopoietin-2 fused to the coiled-coil domain and fibrinogen domain of angiopoietin-1 and that has a Cys to Ser mutation at amino acid 245. (See PCT International Application entitled "Modified TIE-2 Receptor Ligands," published as WO 98/05779 on 12 February 1998 in the name of Regeneron Pharmaceuticals, Inc., especially Figure 27, which is hereby incorporated by reference).

Both angiopoietin-1 and Ang1* require extensive, expensive and labor-intensive purification schemes that result in relatively poor yields of recombinant protein. The need for cost-effective, simple purification schemes for biologicals intended for clinical use can not be over-emphasized.

The COS cell supernatant was purified as described *supra* and yielded approximately 1 mg of purified Ang-1-FD-Fc-FD protein that was used in the studies described *infra* to further characterize the protein.

N-terminal sequencing of COS cell-derived Ang-1-FD-Fc-FD protein: Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. This was of concern because the mutant molecule, Ang1*, has a history of containing between 10-20% N-terminally truncated species. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys. This sequence can be found at amino acids 16-20 of Figure 3A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 Figure 3A.

Receptor binding analysis of COS cell-derived Ang-1-FD-Fc-FD: Previous studies have determined that the fibrinogen domain (FD) of the angiopoietin molecules is necessary for ligand/receptor interaction. Furthermore, in order for high affinity binding to the Tie-2 receptor to

occur, native angiopoietin-1, angiopoietin-2, and the mutant Ang1* must form at least tetrameric, and possibly higher order, multimers. To determine whether the COS cell-derived Ang-1-FD-Fc-FD protein, which is tetrameric with respect to the FD domain, could bind to Tie-2 with high affinity, standard Biacore analysis was performed. Briefly, Tie-2-Fc receptor body protein, which is a fusion protein comprising the ectodomain of Tie-2 fused to the Fc domain of human IgG1, was immobilized on a Biacore chip. Ang-1-FD-Fc-FD-containing solution was passed over the chip and binding between Tie-2 ectodomain and Ang-1-FD-Fc-FD was allowed to occur. The binding step was followed by a 0.5 M NaCl high salt wash. The high salt wash was not able to disrupt the interaction between the Ang-1-FD-Fc-FD protein and the Tie-2 receptor ectodomain, implying that there is a strong interaction between the mutant ligand and receptor. This result is consistent with earlier Biacore results in which both Ang-1-FD-Fc-FD parent molecule, angiopoietin-1 and the mutant Ang1* molecule, have been shown to interact strongly with the Tie-2-Fc receptor and that this interaction is not disrupted by high salt. In contrast, several mutant molecules derived from the parent angiopoietin-1 molecule are readily dissociated from the Tie-2-Fc receptor when treated with high salt. The mutant molecules, designated Ang-1/FD (a monomer with respect to the FD), Ang-1/FD-Fc (also a monomer with respect to the FD, but which is able to form a dimer due to the presence of the Fc domain), and Ang-1/C/FD (a monomer with respect to the FD, but which also contains the coiled-coil domain of angiopoietin-1), do not exist in multimeric forms sufficient for high affinity binding to the Tie-2 receptor.

Example 3: Characterization of COS cell-derived Ang-2-FD-Fc-FD protein.

Molecular Weight Analysis: As described for Ang-1-FD-Fc-FD *supra*, the predicted molecular weight for Ang-2-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form of Ang-2-FD-Fc-FD has a predicted

weight of 76,052, with three predicted N-linked glycosylation sites that could potentially increase the molecular weight of the monomeric protein to 83,552. Like Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,104. SDS PAGE analyses of COS cell-derived protein confirmed these approximate molecular weights, with a band running at about 200kD under non-reducing conditions and a band running at about 88kD under reducing conditions (Figure 7). Light scatter analysis confirmed the molecular weight (171kD) and revealed that the Ang-2-FD-Fc-FD protein, like Ang-1-FD-Fc-FD, exists as a homogeneous species (Figure 8).

Expression Level in COS Cells: COS cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was generated by transiently transfecting COS cells with the Ang-2-FD-Fc-FD DNA construct described *supra*. The COS cell supernatant was analyzed by Biacore to quantitate the amount of Ang-2-FD-Fc-FD protein present in the supernatant. This analysis resulted in an RU value of 606, which is equivalent to 0.7mg of recombinant protein/liter of COS cell supernatant. These values represent relatively high levels of expression.

Purification of COS Supernatants: As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The COS cell supernatant was purified as described for Ang-1-FD-Fc-FD supra and yielded approximately 2 mg of purified Ang-2-FD-Fc-FD protein that was used in the studies described *infra* to further characterize this protein.

N-terminal sequencing: Purified COS cell-derived Ang-2-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Arg-Asp-X-Ala-Glu, wherein X is Cys. This

sequence can be found at amino acids 16-20 of Figure 4A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A.

Receptor binding analysis of COS cell-derived protein: To determine whether the COS cell-derived Ang-2-FD-Fc-FD protein could bind to the Tie-2 receptor, standard Biacore analysis was performed as described for Ang-1-FD-Fc-FD *supra*. As with Ang-1-FD-Fc-FD, a high salt wash was not able to disrupt the interaction between the Ang-2-FD-Fc-FD protein and the Tie-2-Fc receptor, again implying that there is a strong interaction between mutant ligand and receptor.

Example 4: Effects of COS cell-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.

Because Ang-1-FD-Fc-FD is a mutant molecule derived from the agonist angiopoietin-1 and Ang-2-FD-Fc-FD is a mutant molecule derived from the antagonist angiopoietin-2, we wanted to determine whether or not these two mutant molecules would retain the activity associated with the parent molecule from which it was derived.

Assay system: All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA 80:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill in the art.

(A) Ang1*-mediated vs. Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells: EAhy926 cells were stimulated with either 0.1 μg/ml, 0.2 μg/ml, or 0.8 μg/ml Ang1* or Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 9).

(B) Ability of Ang-2-FD-Fc-FD to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells: EAhy926 cells were treated with 0.4 μg/ml of the Tie-2 agonist Ang1* and 1 μg/ml, 2 μg/ml, 4 μg/ml. 6 μg/ ml, or 8 μg/ml of Ang-2-FD-Fc-FD. As shown in Figure 10, Ang-2-FD-Fc-FD is able to block Ang1* stimulation of the Tie-2 receptor when it is present in at least a 10-15 fold molar excess of Ang1*.

(C) Ability of angiopoietin-2 to block Ang1*-mediated Tie-2 receptor phosphorylation in EAhy926 cells: To compare the blocking effects of the naturally occurring antagonist angiopoietin-2 with that of Ang-2-FD-Fc-FD, the same experiment described in (B) *supra* was performed, substituting angiopoietin-2 for Ang-2-FD-Fc-FD. The results of this experiment are shown in Figure 11. At a 20 fold molar excess, the angiopoietin-2 has not reduced the phosphorylation level to 50%. This result, coupled with the results described in (B) *supra* implies that Ang-2-FD-Fc-FD is a more potent inhibitor or Ang1*-mediated Tie-2 receptor phosphorylation than angiopoietin-2.

(D) Ability of Ang-2-FD-Fc-FD to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells: EAhy926 cells were treated with 0.2 μg/ml of the naturally occurring Tie-2 agonist angiopoietin-1 and 1 μg/ml, 2 μg/ml, 4 μg/ml. 6 μg/ ml, or 8 μg/ml of Ang-2-FD-Fc-FD. The results of this experiment, shown in Figure 12, show that while there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, Ang-2-FD-Fc-FD seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 10 and described in (B) *supra*.

(E) Ability of angiopoietin-2 to block angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in EAhy926 cells: EAhy926 cells were treated with 0.2 μg/ml of the angiopoietin-1 and 1 μg/ml, 2 μg/ml, 4 μg/ml,

6 μg/ ml, or 8 μg/ml of angiopoietin-2. The results of this experiment, shown in Figure 13, show that there is a trend toward blocking angiopoietin-1-mediated phosphorylation of the Tie-2 receptor in these cells, but, like Ang-2-FD-Fc-FD, angiopoietin-2 seems to be more effective at blocking Ang1*-mediated phosphorylation of Tie-2, as shown in Figure 11 and described in (C) *supra*.

Example 5: Construction of Ang-1-FD-Fc-FD CHO cell expression vector pRG763/Ang-1-FD-Fc-FD.

The pRG763/Ang-1-FD-Fc-FD CHO cell expression vector was constructed by isolating from the plasmid pCDNA3.1/Ang1-FD-Fc-FD a 2115 base pair EcoRI - NotI fragment containing Ang1-FD-Fc-FD and ligating this fragment into pRG763 vector digested with EcoRI and NotI. A large scale (2L) culture of E. coli DH10B cells carrying the pRG763/Ang-1-FD-Fc-FD plasmid was grown overnight in TB + ampicillin and the plasmid DNA was extracted using a Promega Wizard Plus Maxiprep kit, following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined in a UV spectrophotometer and fluorometer. The plasmid DNA was verified by digestion of aliquots with NcoI and HincII restriction enzymes. All restriction enzyme digest fragments corresponded to the predicted sizes in a 1% agarose gel.

Example 6: Expression of Ang-1-FD-Fc-FD in CHO cells.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763/Ang-1-FD-Fc-FD using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10%

FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and purified as described *infra*.

Example 7: Construction of Ang-2-FD-Fc-FD CHO cell expression vector pRG763/Ang-2-FD-Fc-FD.

The plasmid pRG763/Ang-2-FD-Fc-FD was constructed by isolating from the plasmid pCDNA3.1/Ang-2-FD-Fc-FD a 2097 base pair EcoRI - NotI fragment containing Ang-2-FD-Fc-FD and ligating this fragment into the pRG763 vector digested with EcoRI and NotI. A large scale (1L) culture of E. coli DH10B cells carrying the pRG763/Ang-2-FD-Fc-FD plasmid was grown overnight in TB + ampicillin and the plasmid DNA was extracted using a Promega Wizard Plus Maxiprep kit, following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined in a UV spectrophotometer and fluorometer. The plasmid DNA was also verified by digestion of plasmid DNA with NcoI and Ppu10I restriction enzymes. All restriction enzyme digest fragments corresponded to the predicted sizes in a 1% agarose gel.

Example 8: Expression of Ang-2-FD-Fc-FD in CHO cells.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763/Ang-2-FD-Fc-FD using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after

adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles purified as described infra.

Example 9: Characterization of stable CHO clone-derived Ang-1-FD-Fc-FD protein.

Molecular Weight Analysis: The predicted molecular weight for stable CHO clone-derived Ang-1-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT) The monomeric form (with respect to the Fc) has a predicted weight of 76,349. In addition, there are three predicted N-linked glycosylation sites, approximately 2500 MW/site, that could potentially increase the molecular weight of the monomeric protein to 83,849. Due to the interaction between the Fc components of adjacent molecules, the protein actually exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,698. Subsequent SDS PAGE analyses confirmed these approximate molecular weights, with a band running at about 210kD under non-reducing conditions and a band running at about 85kD under reducing conditions. Light scatter analysis was performed to further confirm the molecular weight and, more importantly, determine whether or not the protein was a homogeneous species. Light scattering is a function of mass and concentration of a macromolecule. To determine molecular weight, the protein sample was injected onto a gel filtration column and the effluent was monitored with an on line light scattering detector and a refractive index and/or a UV detector. The light scattering detector is a MiniDawn laser light scattering detector was from Wyatt Technology

Corporation (Santa Barbara, CA). This instrument measures static light at three different angles. The on line refractive index detector or UV detector serve to measure protein concentration. Astra 4.7 Software (Wyatt Technology Corporation, Santa Barbara, CA) was used to calculate the protein concentration based on either dn/dc (dn = change of refractive index; dc = concentration) or the extinction coefficient of the protein. The molecular weight of protein is then calculated from the angular dependence of light scattering. The results of this analysis show that the dimeric protein appears to be approximately 173.9kD and the presence of a single peak implies that the protein solution is homogenous.

Expression level of Ang-1-FD-Fc-FD in stable CHO clones: CHO cell supernatant containing recombinant Ang-1-FD-Fc-FD protein was generated by stably transfecting CHO cells with the Ang-1-FD-Fc-FD DNA construct described *supra*. The CHO cell supernatant was analyzed by standard ELISA using an anti-human IgG antibody as a capture antibody and an anti-human IgG antibody conjugated to alkaline phosphatase as a reporter antibody to quantitate the amount of Ang-1-FD-Fc-FD protein present in the supernatant. This analysis revealed expression levels of 2-3 pg/cell/day.

Purification of Ang-1-FD-Fc-FD protein derived from stable CHO clone supernatants: Because the Ang-1-FD-Fc-FD protein contains an Fc domain, purification is relatively simple and straight forward using standard Protein A column chromatography (Pharmacia, Inc.) followed by standard size exclusion chromatography (Pharmacia, Inc.). The CHO cell supernatant was purified as described *supra* and the purified ANG-1-FD-Fc-FD protein was used in the studies described *infra* to further characterize the protein.

N-terminal sequencing of stable CHO clone-derived Ang-1-FD-Fc-FD protein: Purified Ang-1-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal

sequence, Arg-Asp-X-Ala-Asp, wherein X is Cys. This sequence can be found at amino acids 16-20 of Figure 3A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 Figure 3A.

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Example 10: Characterization of stable CHO clone-derived Ang-2-FD-Fc-FD protein.

Molecular Weight Analysis: As described for stable CHO clone-derived Ang-1-FD-Fc-FD *supra*, the predicted molecular weight for stable CHO clone-derived Ang-2-FD-Fc-FD protein was determined using the MacVector Program (Kodak, Scientific Imaging Systems, New Haven, CT). The monomeric form of Ang-2-FD-Fc-FD has a predicted weight of 76,052, with three predicted N-linked glycosylation sites that could potentially increase the molecular weight of the monomeric protein to 83,552. Like Ang-1-FD-Fc-FD, the protein exists as a dimer with a predicted molecular weight, including possible N-linked glycosylation, of 167,104. SDS PAGE analyses confirmed these approximate molecular weights, with a band running at about 200kD under non-reducing conditions and a band running at about 85kD under reducing conditions. Light scatter analysis confirmed the molecular weight (176.6kD) and revealed that the stable CHO clone-derived Ang-2-FD-Fc-FD protein, like stable CHO clone-derived Ang-1-FD-Fc-FD, exists as a homogeneous species.

Expression level of Ang-2-FD-Fc-FD derived from stable CHO clones: CHO cell supernatant containing recombinant Ang-2-FD-Fc-FD protein was generated by stably transfecting CHO cells with the Ang-2-FD-Fc-FD DNA construct described *supra*. The CHO cell supernatant was analyzed by standard ELISA using an anti-human IgG antibody as a capture antibody and an anti-human IgG antibody conjugated to alkaline phosphatase as a reporter antibody to quantitate the amount of Ang-2-FD-Fc-FD protein present in the supernatant. This analysis revealed expression levels of approximately 1-2 pg/cell/day.

Purification of stable CHO clone-derived Ang-2-FD-Fc-FD from cell supernatants: As with Ang-1-FD-Fc-FD, Ang-2-FD-Fc-FD protein contains an Fc domain, so purification is relatively simple and straight forward using standard Protein A column chromatography followed by standard size exclusion chromatography. The CHO cell supernatant was purified as described for stable CHO clone-derived Ang-1-FD-Fc-FD *supra* and was used in the studies described *infra* to further characterize this protein.

N-terminal sequencing of stable CHO clone-derived Ang-2-FD-Fc-FD protein: Purified stable CHO clone-derived Ang-2-FD-Fc-FD protein was subjected to standard N-terminal sequence analysis to determine if any truncated species of the protein were being generated. The analysis revealed only one N-terminal sequence, Asp-X-Ala-Glu-Val, wherein X is Cys. This sequence can be found at amino acids 17-21 of Figure 4A, and immediately follows the protein's signal sequence corresponding to amino acids 1-15 of Figure 4A.

Example 11: Effects of stable CHO clone-derived Ang-1-FD-Fc-FD and Ang-2-FD-Fc-FD on Tie-2 receptor phosphorylation in EAhy926 cells.

Assay system: All of the experiments described *infra* utilized the cell line EAhy926 (Edgell, C. J., et al., (1983) Proc. Natl. Acad. Sci. USA 80:3734-3737) and standard phosphorylation assays and reagents familiar to those of skill in the art.

(A) Ang1*-mediated vs. stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells: EAhy926 cells were stimulated with 0.4 μg/ml Ang1* or 0.2 μg/ml or 0.4 μg/ml stable CHO clone-derived Ang-1-FD-Fc-FD protein. A standard phosphorylation assay revealed that or stable CHO clone-derived Ang-1-FD-Fc-FD was equivalent to Ang1* in its ability to stimulate phosphorylation of the Tie-2 receptor in EAhy926 cells (Figure 17).

(B) Ability of stable CHO clone-derived Ang-2-FD-Fc-FD to block stable CHO clone-derived Ang-1-FD-Fc-FD-mediated Tie-2 receptor phosphorylation in EAhy926 cells: EAhy926 cells were treated with 0.2 μg/ml of the Tie-2 agonist Ang-1-FD-Fc-FD and 2 μg/ml, 4 μg/ml, 8 μg/ml or 16 μg/ml of stable CHO clone-derived Ang-2-FD-Fc-FD. As shown in Figure 18, Ang-2-FD-Fc-FD is able to block stable CHO clone-derived Ang-1-FD-Fc-FD stimulation of the Tie-2 receptor when it is present in at least a 40 fold molar excess of stable CHO clone-derived Ang-1-FD-Fc-FD.

Ephrin ligands:

In previous experiments (Davis et al., 1994, Science, 266:816-819; Gale et al., 1996, Neuron 17:9-19, Gale and Yancopoulos, 1997, Cell Tissue Research 290:227-241), soluble, unclustered Ephrin-B1-Fc and Ephrin-B2-Fc, which dimerize at their respective Fc domains and therefore are dimeric with respect to either the Ephrin-B1 or Ephrin-B2 ectodomain, failed to induce EphB2 receptor phosphorylation. However, when either molecule was multimerized by pre-clustering with an anti-Fc antibody, they became potent agonists for the EphB2 receptor, as judged by tyrosine phosphorylation of the EphB2 receptor in a COS cell reporter assay. Because multimerization of both Ephrin-B1 and Ephrin-B2 appears to be necessary for induction of receptor phosphorylation, we theorized that a molecule that contained tandem repeats of either Ephrin-B1 or Ephrin-B2 ectodomains fused to an Fc domain, which would be dimeric with respect to the Fc domain but which would be tetrameric with respect to Ephrin ectodomains, might be sufficiently clustered to induce receptor phosphorylation. To test this hypothesis, the following DNA constructs were constructed, recombinant proteins produced, and reporter assays performed.

Construction of tandem Ephrin ectodomain/Fc domain nucleic acid molecules.

All of the following nucleic acid molecules were constructed by standard recombinant DNA techniques (See e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), sequence-verified by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), and subcloned into either the mammalian expression pJFE14 (Ephrin-B1-Ephrin-B1-Fc) or pMT21 (Ephrin-B2-Ephrin-B2-Fc), each with a Kozak sequence (Kozak, M., 1987, Nucleic Acids Res. 15:8125-8148) at the 5' end to promote protein translation. The bridging sequences described *infra* were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains, but there is no indication that there is a very critical nature to these bridging sequences (though varying the length of the linker in some of these constructs led to some variation in the amount of protein produced).

Example 12: Construction of Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules.

(A) Ephrin-B1-Ephrin-B1-Fc: The Ephrin-B1-Ephrin-B1-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B1 (Davis et al., ibid.), which corresponds to nucleotides 1-711 of Figure 14A-Figure 14B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 712-720 of Figure 14B), followed by a second copy of the ectodomain of Ephrin-B1 (corresponding to nucleotides 721-1344 of Figure 14B-Figure 14D), except that in this copy of the Ephrin-B1 ectodomain the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1345-1353 of Figure 14D),

followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1354-2049 of Figure 14D-Figure 14E).

(B) Ephrin-B2-Ephrin-B2-Fc: The Ephrin-B2-Ephrin-B2-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann et al., 1995, Mol. Cell Biol. 15:4821-4929), which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E).

As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.

Example 13: Expression of tandem Ephrin recombinant proteins in COS cells.

COS cells were transiently transfected with either the Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc nucleic acid molecules described *supra* using standard transfection techniques known in the art. Two days subsequent to transfection, the growth medium (DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% calf serum) was aspirated and replaced with serum-free medium (DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine). Cell were grown for an additional three days and then the serum-free medium containing the recombinant proteins was collected. Recombinant protein concentration was determined by performing dot blots and comparing the

signal obtained to a standard curve. Once approximate protein concentrations were determined, the Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc recombinant proteins were used in the cell reporter assays described *infra*.

Example 14: Characterization of the COS cell-derived tandem Ephrin ectodomain/Fc domain recombinant proteins.

Reporter Assay: COS cells, which endogenously express the Eph family receptor EphB2 (Gale et al., 1996, Neuron 17:9-19), were used in reporter assays to evaluate the ability of Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc to induce receptor phosphorylation. The assays were performed as previously described (Davis et al., ibid.; Gale et al., ibid.). Briefly, COS cells were grown to 80-90% confluency in standard growth medium described *supra*. After growth, the medium was aspirated, and replaced with serum-free medium (described *supra*) for 1-2 hours prior to treatment with either Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc recombinant protein. The cells were stimulated with 500 ng/ml Ephrin-B1-Ephrin-B1-Fc or Ephrin-B2-Ephrin-B2-Fc for 30 minutes at 37°C, with or without affinity purified human IgG1 Fc-specific goat anti-human antibody (Jackson Immunoresearch, West Grove, PA) at a final concentration of 17 µg/ml. This antibody is capable of clustering the Fc tagged fusion. Subsequent to treatment, the COS cells were harvested and cell lysates were prepared as described in Davis, et al. and Gale, et al., *supra*. The EphB2 receptor protein was immunoprecipitated from the cell lysates using an anti-EphB2 antisera (Henkemeyer et al., 1994, Oncogene 9:1001-1014). Immunoprecipitates were resolved by standard SDS PAGE and transferred to PVDF membranes (Millipore) for western blot analysis. The membranes were probed with either anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Institute, Lake Placid, NY) or anti-EphB2 antibodies (Henkemeyer, et al., ibid.) to determine the extent of EphB2

phosphorylation and the relative quantities of EphB2 in the experimental conditions described *supra*.

Results: Both Ephrin-B1-Ephrin-B1-Fc and Ephrin-B2-Ephrin-B2-Fc were shown to be approximately as active as anti-Fc antibody-clustered Ephrin-B1-Fc in their ability to induce EphB2 receptor phosphorylation in the COS cell reporter assay. Furthermore, if either of the proteins were further clustered with the goat anti-human Fc antibody, they became even more potent in their ability to induce EphB2 receptor phosphorylation. Figure 16 shows the results of this phosphorylation assay.

Example 15: Construction of Ephrin-B2-Ephrin-B2-Fc CHO expression vector.

The Ephrin-B2-Ephrin-B2-Fc DNA molecule consists of the coding sequence of the ectodomain of Ephrin-B2 (Bergemann et al., 1995, Mol. Cell Biol. 15:4821-4929), which corresponds to nucleotides 1-675 of Figure 15A-Figure 15B, followed by a bridging sequence consisting of the amino acids Gly-Pro-Gly (nucleotides 676-684 of Figure 15B), followed by a second copy of the ectodomain of Ephrin-B2 (corresponding to nucleotides 685-1270 of Figure 15B-Figure 15D), except that in this copy the signal sequence has been removed. This second copy is followed by a second Gly-Pro-Gly amino acid bridge (nucleotides 1270-1278 of Figure 15D), followed by the coding sequence for the Fc portion of human IgG1 (nucleotides 1279-1977 of Figure 15D-Figure 15E). This molecule was subcloned into the HindIII and NotI polylinker sites in the expression vector pRG763 and was designated pRG763-m(Ephrin-B2)2-Fc. As with the angiopoietin nucleic acid molecules described *supra*, the bridging sequences were introduced to provide convenient restriction sites and to give flexibility to the junctions between the domains.

Example 16: Expression of Ephrin-B2-Ephrin-B2-Fc in CHO-K1 (E1A) cells.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pRG763-m(Ephrin-B2)2-Fc using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and purified as described *supra*.